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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No.	ET 390094660 US

INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
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Pierre J.	Verroust	Paris, France
Patricia L.	Allen	New Orleans, LA

☑ *Additional inventors are being named on the* 1 *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

Treatments for Cancer and Other Conditions

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Specification *Number of Pages* 32 ☐ CD(s), Number _____

☑ Drawing(s) *Number of Sheets* 6 ☐ Other (specify) _____

☐ Application Data Sheet. See 37 CFR 1.76

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Respectfully submitted,

SIGNATURE Harl R. Tolbert

Date 01/23/2004

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Additional Page

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INVENTOR(S)/APPLICANT(S)		
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Timothy G.	Hammond	New Orleans, LA

Number 1 of 1

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FEE TRANSMITTAL for FY 2002

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TOTAL AMOUNT OF PAYMENT ($) 80.00

Complete if Known	
Application Number	USPS Express ET 390094660 US
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First Named Inventor	Timothy Hammond
Examiner Name	
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Attorney Docket No.	TM-284

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101	740	201	370	Utility filing fee	
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107	510	207	255	Plant filing fee	
108	740	208	370	Reissue filing fee	
114	160	214	80	Provisional filing fee	80.00

SUBTOTAL (1) ($) 80.00

2. EXTRA CLAIM FEES

		Extra Claims		Fee from below	Fee Paid
Total Claims		-20** =		X	=
Independent Claims		- 3** =		X	=
Multiple Dependent					=

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
103	18	203	9	Claims in excess of 20
102	84	202	42	Independent claims in excess of 3
104	280	204	140	Multiple dependent claim, if not paid
109	84	209	42	** Reissue independent claims over original patent
110	18	210	9	** Reissue claims in excess of 20 and over original patent

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FEE CALCULATION (continued)

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127	50	227	25	Surcharge - late provisional filing fee or cover sheet	
139	130	139	130	Non-English specification	
147	2,520	147	2,520	For filing a request for *ex parte* reexamination	
112	920*	112	920*	Requesting publication of SIR prior to Examiner action	
113	1,840*	113	1,840*	Requesting publication of SIR after Examiner action	
115	110	215	55	Extension for reply within first month	
116	400	216	200	Extension for reply within second month	
117	920	217	460	Extension for reply within third month	
118	1,440	218	720	Extension for reply within fourth month	
128	1,960	228	980	Extension for reply within fifth month	
119	320	219	160	Notice of Appeal	
120	320	220	160	Filing a brief in support of an appeal	
121	280	221	140	Request for oral hearing	
138	1,510	138	1,510	Petition to institute a public use proceeding	
140	110	240	55	Petition to revive - unavoidable	
141	1,280	241	640	Petition to revive - unintentional	
142	1,280	242	640	Utility issue fee (or reissue)	
143	460	243	230	Design issue fee	
144	620	244	310	Plant issue fee	
122	130	122	130	Petitions to the Commissioner	
123	50	123	50	Processing fee under 37 CFR 1.17(q)	
126	180	126	180	Submission of Information Disclosure Stmt	
581	40	581	40	Recording each patent assignment per property (times number of properties)	
146	740	246	370	Filing a submission after final rejection (37 CFR § 1.129(a))	
149	740	249	370	For each additional invention to be examined (37 CFR § 1.129(b))	
179	740	279	370	Request for Continued Examination (RCE)	
169	900	169	900	Request for expedited examination of a design application	

Other fee (specify) _____

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SUBMITTED BY				Complete (if applicable)	
Name (Print/Type)	Harl Tolbert	Registration No. (Attorney/Agent)		Telephone	(504) 585-6962
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UNITED STATES PROVISIONAL PATENT APPLICATION

Title of the Invention
"Treatments for Cancer and Other Conditions"

INEVENTORS
R. Bryan Klassen, a United States citizen of Harahan, Louisiana
Pierre J. Verroust, a French citizen of Paris, France
Patricia L. Allen, a United States citizen of New Orleans, Louisiana
Timothy G. Hammond, a United States citizen of New Orleans, Louisiana

STATEMENT REGARDING GOVERNMENT SUPPORT

This work was supported by NIEHS ARCH ES09996 (R. Klassen and T. Hammond).

FIELD OF THE INVENTION

The present invention relates to methods of reducing the toxicity of heavy metal treatments for cancer and other ailments treated with heavy metals.

BACKGROUND

Renal exposure to heavy metals and the resultant toxicity is often mediated by the protein metallothionein. Produced by several tissues and able to circulate, metallothionein participates whether the metal's origin is environmental and occupational (cadmium and mercury), genetic (copper), or therapeutic (gold and platinum). For example, environmental and occupational exposure to cadmium is widespread but mostly chronic and low-level (1 ATSDR). Whether ingested or inhaled, the majority of absorbed cadmium eventually complexes with metallothionein (2 Nordberg, 3 Chan). The resulting Cd metallothionein complex is small enough (~7 kDa) to be freely filtered through the renal glomerulus into the proximal tubular fluid before reuptake into proximal tubular cells (4 Foulkes). Although neither the apoprotein nor the zinc complex appears toxic, the heavy metal complex Cd metallothionein is a renal tubular toxin whose damage is marked by proteinuria, glucosuria, and aminoaciduria, or in more severe cases, acute tubular necrosis or chronic renal failure (5 Rehm).

Metallothionein metabolism is also critical in both physiology and therapeutics because heavy metals have a narrow margin between their essential or useful and their toxic levels (6 Suzuki). Mutations of metallothionein are associated with several copper storage diseases (6 Suzuki, 7 Okabe). Metallothionein also carries the commonly prescribed therapeutics gold and cisplatinum: both of these are limited in acute dose selection and duration of chronic therapy by nephrotoxicity based in the renal proximal tubule (Platinum: 8 Ramesh & Reeves, 9 Boogaard; gold: 10 Saito, 11 Saito, 12 Glennas).

In all these cases, the major site of renal damage is the proximal tubule, highlighting the importance of this tissue as a target for therapy. Free cadmium, copper and cisplatinum are toxic predominantly in the S_3 proximal straight tubule (42 Sabolic, 8 Ramesh, 7 Okabe), while conjugation to metallothionein shifts the damage to S_1 and S_2 sub-segments in the renal cortex

(47,7). In the case of the therapeutic heavy metals gold and cisplatinum, inhibition of renal uptake is desirable to enable the broadening of dose selection and treatment duration. Similarly, cadmium turnover in the body suggests that an appropriate therapy aimed at urinary excretion, secondary to inhibition of renal proximal tubular uptake of Cd metallothionein, could eliminate much of the accumulation.

While heavy metals disturb many functions within the proximal tubules, the entry route of heavy metal-metallothionein complexes into the epithelial cells remains unknown (13 Nordberg, 14 Fowler, 15 Liu, 16 Endo, 17 Kroning, 18 Sharma, 19 Kone). Conflicting reports implicate different transporters or receptor mediated pathways (4 Foulkes, 20 Bernard, 21 Bernard, 22 Tsuruoka, 23 Kinne, 24 Marshall). At least some of the uncertainty arises from the use of in vivo and in vitro models that differ significantly in their behavior. For example, while $CdCl_2$ is more toxic than Cd metallothionein to cultured rat kidney proximal tubules and LLC-PK1 cells, Cd metallothionein shows greater in vivo nephrotoxic effects (25 Liu, 26 Prozialeck, 42 Sabolic). The lack of consensus complicates the search for a therapy for renal heavy metal poisoning. Identifying the entry step, critical to the design of protective agents, was the main objective of the research reported here.

Genetic mutations of metallothionein, leading to reduced copper binding, are implicated in several copper storage diseases (6 Suzuki, 7 Okabe). Heavy metals used therapeutically are limited by their renal toxicity. Gold therapy is predominantly used to treat rheumatoid arthritis, but the duration and dosage of gold administration are limited by nephrotoxicity (10 Saito, 11 Saito, 12 Glennas). Cisplatinum, one of the most commonly prescribed chemotherapeutic agents, contains the heavy metal platinum, and is also restricted in dose and duration of therapy by renal tubular damage (8 Ramesh & Reeves, 9 Boogaard).

Conjugation of heavy metals to metallothionein changes the nephron site of renal uptake of the heavy metals and the degree of toxicity. Okabe et al (7 Okabe) were the first to demonstrate changes in the nephron site of uptake of heavy metals following conjugation to metallothionein. They observed that free Cu was taken up in the inner medulla of mice, but pre-

conjugation of the Cu to metallothionein changed the uptake to the cortex of the kidney. Sabolic et al (42 Sabolic) made the same observation with cadmium, and added careful morphological analysis of the nephron segments from S_3 for un-conjugated cadmium to peri-glomerular S_1 for metallothionein conjugated cadmium. The dramatic element in this analysis was the finding that metallothionein conjugation of cadmium increased the tubular toxicity enormously.

In order to determine the mechanisms of nephrotoxicity of heavy metals and to design appropriate protective strategies, it is vital to identify the pathway by which metallothionein enters proximal tubular cells. The observations that metallothionein interferes with the uptake of β_2-microglobulin in conscious rats (20 Bernard, 21 Bernard) and that β_2-microglobulin is predominantly taken up by binding to megalin (28 Verroust) are consistent with the conclusion that metallothionein is a ligand for megalin. The distribution and molecular characteristics of megalin are consistent with the observations on metallothionein uptake: megalin is the most abundant protein in the proximal tubule of the kidney, it is heavily expressed in S_1 in the peri-glomerular area, and it is a multi-ligand receptor with multiple binding sites.

The inventors observed that megalin or cubilin might be involved in metallothionein uptake because these scavenger receptors mediate the proximal tubular uptake of many ligands with quite different properties. Megalin binds not only proteins like β_2-microglobulin, cytochrome C, and retinol-binding protein, but also polybasic antibiotics such as gentamicin (27 Verroust, 28 Verroust, 29 Moestrup, 30 Saito). Cubilin, the other abundant proximal tubular receptor, also has diverse ligands (31 Birn). Interestingly, proximal tubular uptake of metallothionein and of β_2-microglobulin is mutually inhibitory in conscious rats (20 Bernard). Taken together with the observation that megalin mediates uptake of β_2-microglobulin, this result provides indirect evidence implicating megalin in metallothionein uptake. As all these ligands

are freely filtered by the glomerulus, they are available in the proximal tubule for competition with metallothionein to act as protective agents, if we can demonstrate that inhibition observed in direct molecular interactions matches the whole animal pathophysiology. The inventors report here that megalin binds metallothionein and implicates the highly conserved hinge or interdomain region of metallothionein, centered on a lysine repeat, as the critical site for binding to megalin. The discovery of mutants of metallothionein that bind heavy metals, but do not bind megalin, and thus are not taken up by proximal tubular cells, offers great hope in the development and use of heavy metal therapeutics.

DESCRIPTION OF FIGURES

Figure 1. Dose-dependent binding of metallothionein-I to megalin. Rabbit kidney metallothionein-I in HBS containing 2 mM Ca and Mg. (a) From bottom to top, the traces represent the responses obtained with 75, 150, 300, 600, 1200, and 2400 ug/mL metallothionein. (b) Fit of the maximum responses obtained after 2.5 minutes. The double referencing method of Myszka (38, Myszka) was used to eliminate artifacts in the data.

Figure 2. Displacement of fluorescently labeled metallothionein-I from brush border membranes by anti-receptor antisera. Brush border membrane vesicles isolated from rat renal cortex were incubated with fluorescently conjugated metallothionein and receptor antisera. The observed fluorescence is shown for the control (metallothionein alone) and metallothionein in the presence of anti-cubilin, anti-megalin, and anti-NK1-peptide antibodies. Data files of 2000 observations per sample were collected.

Figure 3. Time-dependent uptake of fluorescently labeled metallothionein-I in BN-16 cells and colocalization with megalin and cubilin (5 and 15 min). Samples were incubated with labeled metallothionein-I for 1 hr at 4 °C before exchange with unlabeled metallothionein-I and incubation at 37°C for 5 or 15 min. (a) metallothionein-I uptake after 5 min. (b) Megalin after 5 min. (c) Dual wavelength exposure of metallothionein-I and megalin after 5 min. (d) metallothionein-I uptake after 15 min. (e) Megalin after 15 min. (f) Dual wavelength exposure of metallothionein-I and megalin after 15 min. At both times, cubilin yielded similar results. (g) metallothionein-I uptake in a single cell after 15 min, viewed by using confocal microscopy. (h) Megalin in a single cell after 15 min, viewed by using confocal microscopy. (i) Dual wavelength visualization of metallothionein-I and megalin in a single cell after 15 min, viewed by using confocal microscopy. The views in panels (a) – (f) are scaled differently than panels (g) – (i); the latter focus on a single cell.

Figure 4. metallothionein-I sequence, cadmium binding sites and domains, and peptide fragments used in interference studies. The sequence of metallothionein-I is shown in the center. The cysteine residues that bind seven cadmium ions, and also the two domains containing them, are indicated below the sequence. The six peptides used in the preliminary interference studies are shown at the top. At the bottom, the lysine repeat that appears in the hinge region and also in the interfering peptide SCKKSCC is shown. (Adapted with additions from 51, Kägi)

Figure 5. Binding of hinge peptide SCKKSCC and altered binding of metallothionein-I to megalin in the presence of the peptide. (a) Hinge peptide SCKKSCC in HBS containing 2 mM Ca and Mg. From bottom to top, the traces represent the responses obtained with 63, 125, 250, and 500 ug/mL peptide. Each trace represents the average of three replicates and was corrected by referencing to blank buffer injections. (b) Responses obtained when metallothionein-I was injected alone and in the presence of hinge peptide SCKKSCC. The upper trace shows the response when metallothionein-I is injected at a concentration of 2000 ug/mL. The lower trace shows the response when metallothionein-I (2000 ug/mL) and peptide (250 ug/mL) are coinjected. Each trace represents the average of three replicates and was corrected by referencing to blank buffer injections.

Figure 6. Fluorescein-Metallothionein uptake. The results shown in the figure above show that compared to a non-fluorescent control (0±0 arbitrary fluorescent units, n=6), the cell took up fluorescent-MT (125±18, n=6, $p<0.01$). Both full length recombinant MT (13±1) and alpha subunit with SCMKSCC intact (5±1) significantly inhibited uptake of fluorescent MT (n=6, $p<0.01$ ANOVA and Scheffe). The beta subunit recombinant in which SCKKSCC is disrupted had significantly less effect (64±5, n=6, $p<0.05$).

SUMMARY OF THE INVENTION

The inventors provide three lines of evidence that megalin binds metallothionein, and that megalin is by far the most quantitatively important mechanism of metallothionein uptake into the renal proximal tubule. First, SPR directly demonstrates binding of the purified proteins in a dose, ion and pH dependent manner. Second, antibody interference experiments show that >90% of the metallothionein binding on brush border membrane vesicles can be displaced specifically with anti-megalin, but not control anti-cubilin or anti-NK-1 receptor, antisera. Finally, megalin and metallothionein co-localize at the cellular level in fluorescent microscopy studies. Megalin and metallothionein co-localize and internalize concomitantly before separating in the late endosomal pathway. We present several lines of evidence indicate that megalin is the receptor responsible for the uptake of Cd metallothionein in the proximal convoluted tubules.

The present invention provides mutants and fragments of metallothionein that bind a variety of metals, including heavy metals but do not bind megalin and are not taken up by cells of the kidney, including proximal tubular cells. The present invention provides mutants and fragments of metallothionein that bind a variety of metals, including heavy metals but do not bind megalin and are not taken up by cells of the ear or inner ear. The present invention provides mutants and fragments of metallothionein that bind a variety of metals, including heavy metals but do not bind megalin and are not taken up by other cells of the body.

DETAILED DESCRIPTION OF THE INVENTION

These studies used commercially available metallothionein-I, a highly conserved mammalian isoform of metallothionein. All known metallothionein-I sequences contain 61 or 62 amino acids with 20 conserved cysteine residues and are able to bind up to seven equivalents of divalent metal ions (40 Huang), commonly a mixture of zinc and cadmium. Although Zn metallothionein and Cd metallothionein differ dramatically in their toxic effects, they produce virtually identical profiles in their binding and uptake (41 Dorian). In solution, metallothionein-I tends to form oligomers (43 Tang). Therefore, metallothionein-I was used as received, rather than saturated with cadmium in an extra step, to maximize the structural integrity of the metalloprotein during our analyses.

Surface plasmon resonance experiments.

To determine which receptors might bind metallothionein, we immobilized and studied megalin and cubilin individually by using SPR. Binding to a single site on megalin was dose dependent over a 32-fold change in concentration range (Figure 1). The estimated dissociation constant, 9.8×10^{-5} M, may appear small for a receptor-ligand interaction, but is similar to values obtained for other known megalin ligands (45 Gburek). In contrast, no binding of metallothionein-I to cubilin was observed. The calcium dependence of metallothionein-I binding is also consistent with similar ion requirements of other megalin ligands (31 Birn, 32 Moestrup), but the dependence on magnesium is unusual.

Data from SPR experiments can be significantly affected by a variety of events, especially non-specific binding of solutes and analyte to the receptor or to the dextran matrix hosting it. The acquisition of meaningful data frequently requires the use of a reference matrix.

In our studies, ovalbumin, casein, or transferrin was used to prepare a negative control and also to eliminate experimental artifacts inherent in SPR according to the method of Myszka (37 Myszka).

Flow cytometry experiments.

Megalin does not retain its full structural and functional integrity long in a membrane-free environment at room temperature. Conditions required for release of this integral membrane protein use Triton-X, and the modest changes in pH must also be of limited duration. Fortunately megalin and cubilin retain their structural and functional integrity in brush border membrane vesicles, which can be readily obtained from cortical tissue. These renal vesicles retain a significant number of proteins whose behavior can be analyzed by flow cytometry using antibodies to probe molecular binding events. Fluorescent metallothionein bound to vesicles (Figure 2). As with the SPR experiments, there were clear differences in the behavior of megalin and cubilin in vesicles toward metallothionein. Anti-megalin antibodies displaced nearly all bound metallothionein. In stark contrast, antibodies to cubilin or to the unrelated NK-1 receptor had no statistically significant effect on metallothionein binding.

Fluorescence microscopy experiments using cultured cells.

The problems attending the isolation of megalin can be sidestepped altogether by studying the receptor using cultured cells. Unfortunately, traditional renal cell lines such as OK and LLC-PK1 do not express cubilin and megalin well in culture. Instead, we chose to use an immortalized line of yolk sac cells of Brown Norway rats (BN-16). These cells have previously been shown to express both cubilin and megalin abundantly, and to share many structural and

biochemical features of renal proximal tubular cells including the distinctive microvilli, early endosomal vesicles and vacuoles, and dense apical tubules (38 Le Panse).

The results are consistent with an endocytic process such at that involving megalin and cubilin. When cells are maintained at temperatures too low to permit appreciable endocytosis, metallothionein-I binds to the surface but does not enter the cells (Figure 3, panels a-c). Incubating these cells at 37 °C afterward leads to uptake. After only 15 min at 37 °C, some metallothionein-I has already been internalized and most is co-localized with megalin and cubilin (Figure 3, panels d-f). That some of the receptor is free of associated metallothionein-I is to be expected: these receptors are internalized by a clathrin-dependent process into the early endosomes, discharge their ligands, and are subsequently recycled to the surface (28 Verroust). Thus, these receptors are normally found both within the cell and on the cell surface.

Because metallothionein-I is very small, some diffusion of the protein may occur over time in samples even after the cells are fixed, or the fluorophore may be cleaved from the carrier protein, creating diffuse fluorescence. This complication can be addressed by comparing the results with those found when antibodies to unrelated proteins are used. The results found for megalin and cubilin bear no resemblance to those for giantin, an unrelated protein found in the Golgi apparatus.

Preliminary studies using confocal microscopy confirm that metallothionein-I and megalin or cubilin are co-localized. In panels g-i of Figure 3, similar patterns of distribution for fluorescent metallothionein-I and antibody are clearly visible within the cell shown. While protein diffusion or fluorophore cleavage contributes significantly to background, regions of high concentration of metallothionein-I are invariably associated with a similar concentration of receptor.

Animals, reagents and antibodies.

Male Sprague Dawley rats (200 - 250 gm) were obtained from Sasco, Omaha, NE. All reagents were from Sigma Chemicals (St. Louis MO) unless otherwise stated. Metallothionein class I (metallothionein-I) isolated from either rabbit liver or horse kidney was used as received. The supplier reported metal assays of metallothionein samples show ~ 7% metals by mass, which indicated complete occupation of all metal-binding sites by zinc and/or cadmium. Purified human megalin and cubilin receptors were obtained by detergent solubilization of renal cortex brush border membranes followed by affinity chromatography using immobilized receptor-associated protein (32 Moestrup). Polyclonal antibodies against cubilin and megalin, and transferrin were raised against proteins purified by immunoaffinity chromatography using previously reported monoclonal antibodies coupled to Sepharose 4B (32 Moestrup, 33 Hammond, 34 Sahali, 35 Sahali). These antibodies were monospecific by immunoblotting on whole brush border preparations and by immunoprecipitation of biosynthetically labeled yolk sac epithelial cells in culture (34 Sahali, 35 Sahali). Anti-neurokinin-1/substance-P receptor antiserum was a kind gift of Dr. Jacques Couraud, Gif-sur-Yvette, France (36 Bret-Dibat). Anti-giantin was kindly provided by H .P. Hauri. Fluorescent secondary antibodies were obtained from Dako (mouse FITC-anti-goat antibody) or from Molecular Probes (goat anti-mouse, goat anti-rabbit, and donkey anti-sheep antibodies, all conjugated to Alexa 488.

Surface plasmon resonance (SPR) experiments.

The interaction of metallothionein-I with megalin was assayed with a BIACORE 3000 biosensor system (Biacore AB). In SPR, one protein is immobilized to a dextran-coated gold

surface. Injection of a soluble protein produces a signal change that is directly proportional to the mass of bound protein and is reported as resonance units (RU). Megalin (0.025 mg/mL in 10 mM acetate, pH 4.53) was immobilized (1000-3000 RU) in one flow cell on a CM5 biosensor chip using standard primary amine-coupling methods as detailed by the manufacturer. An equal amount of either ovalbumin or casein was immobilized in a second flow cell to provide real-time reference correction for instrumental artifacts and non-specific binding events. Rabbit liver metallothionein-I was injected over both flow cells at room temperature in HEPES-buffered saline (HBS), pH 7.4, containing 2 mM Ca, and 2 mM Mg, and 0.005% surfactant P20. Maximum reproducibility was obtained when 0.0008% sodium dextran sulfate (Pharmacia Biotech, cat. no. 17-0340-01) was also included in the buffer. Equilibrium dissociation constants (K_d) were determined from steady-state binding measurements at concentrations ranging from 75 to 2400 ug/mL. Proteins were typically injected at flow rates of 50 uL/min for 3 min and then allowed to dissociate for 5 min. Because metallothionein-I is a low-affinity ligand, no regeneration (removal of bound protein by injection of a second, typically harsh, solvent) was necessary. The "double-referencing" technique of Myszka (37 Myszka) was used to eliminate additional instrumental artifacts; the results are reflected in Figure 1. Thermodynamic constants were calculated using Biacore's BIAevaluation 3.1 software. The blank injections used for this procedure were identical to sample solutions except for the omission of metallothionein-I.

Peptide inhibition studies.

Six 16-amino acid peptides spanning the entire metallothionein-I sequence were obtained from Biosource International. In preliminary studies of peptide binding to megalin, the peptide concentrations were approximately 5 uM, while rabbit liver metallothionein-I was approximately

250 ug/mL, corresponding to K_d(estimated). The SCKKSCC peptide, representing the overlap sequence between two of these peptides, was also obtained from Biosource International. Megalin was immobilized as described above. An equal amount of transferrin (0.10 mg/mL in 10 mM acetate, pH 4.96) was immobilized in a second flow cell to provide real-time reference correction. Dose dependent peptide binding was examined by injecting the peptide at concentrations ranging from 0 to 500 ug/mL. Inhibition of metallothionein-I binding by peptide was examined by injecting rabbit liver metallothionein-I as described above and comparing the results to samples that contained varying concentrations of peptide but were otherwise identical. No regeneration was necessary. Additional artifacts were eliminated before curve-fitting by applying double referencing techniques. The blank injections used for this procedure were identical to sample solutions except for the omission of metallothionein-I and peptide.

Preparation of fluorophore-conjugated metallothionein-I.

Metallothionein-I was conjugated to Alexa Fluor 594 (Molecular Probes) following the supplier's protocols. Because metallothionein-I is a very small protein, unreacted dye was removed by dialysis against PBS at pH 7.4 in Slide-A-Lyzer dialysis cassettes having 3500 Da MWCO (Pierce, cat. no. 66330).

Cell culture studies.

Except as noted, experiments were conducted using immortalized yolk sac cells from the Brown Norway rat (BN-16) (38 Le Panse). An apical brush border and a specialized endosomal pathway similar to the renal proximal tubule, including abundant expression of megalin and cubilin, characterize these cells. The cells were grown in Dulbecco's Modified Eagle's Medium

(DMEM; GIBCO) supplemented with 10% fetal calf serum and 50 ug/mL streptomycin. Cells were passaged every four days with a split ratio of 10:1. MDCK cells were grown in a modified minimal essential medium as described in ATCC protocols.

Metallothionein-I uptake by BN-16 cells. Uptake experiments were performed with confluent monolayers cultured in eight-chamber glass slides (Nalge Nunc International, Naperville, IL). The BN-16 cells were cultured on chambered slides until confluent (approximately 10-18 hours). The monolayers were washed twice with cold PBS and allowed to equilibrate at 4 °C in a cold room. The labeled metallothionein-I in DMEM containing 0.01% ovalbumin was added at concentrations ranging from 0.075 to 12 uM. After incubation at 37 °C for 20 min, the medium was removed and the cells were washed successively with PBS/0.1% ovalbumin (2X) and PBS before fixing and mounting. The slides were examined by use of a fluorescence microscope (Leica DMR) equipped with a color video camera (Sony 3CCD). This experiment was used to select a concentration of 1.0 uM for subsequent experiments involving the labeled ligand.

In a time-dependent uptake experiment using labeled metallothionein-I, cells were prepared as before but incubated with 1.0 uM ligand for intervals of 5, 15, 30, and 45 min.

In receptor colocalization experiments, the cells were permeabilized with Triton X-100 (0.05% in PBS) and treated with the appropriate primary and secondary antibodies after fixation. The primary antibodies included anti-megalin, anti-cubilin, anti-TfR, and anti-giantin.

In order to follow the internalization of metallothionein, Alexa-labeled metallothionein-I was added at concentrations of 1.0 or 6.0 uM and the cells were incubated in the cold for intervals ranging from 5 to 45 min before fixing. Based on these experiments, confluent monolayers were washed with PBS and allowed to equilibrate in a cold room with labeled

metallothionein-I (2.5 uM) for one hr at 4 °C. After washing with PBS, the cells were treated with warm DMEM containing 2.5 uM unlabeled metallothionein-I and 0.01% ovalbumin, and immediately transferred to an incubator. Cells were fixed at intervals of 5, 15, and 45 min. Finally, the cells were permeabilized and incubated with the appropriate primary and secondary antibodies to localize megalin, cubilin, and TfR.

Metallothionein-I uptake by MDCK cells. MDCK cells were cultured on chambered slides until confluent (approximately 2 days). The monolayers were washed twice with PBS and treated with labeled metallothionein-I in DMEM containing 0.01% ovalbumin. The labeled metallothionein-I was added at a concentration of 1.0 uM. After incubation at 37 °C for 30 min, the medium was removed and the cells were washed successively with PBS/0.1% ovalbumin (2X) and PBS before fixing and mounting. To assist in visualization of the cells, some samples were permeabilized with Triton X-100 (0.05% in PBS) and stained with DAPI.

The slides were examined by use of a fluorescence microscope (Leica DMR) equipped with a color video camera (Sony 3CCD). Confocal microscopy was carried out with a Leica TCS equipped with a DMR inverted microscope and a 63/1.4 objective. Image processing was performed with the use of the Leica's online Scanware software. Numeric images were processed with the use of Scion Image and Photoshop 5.0 software.

Uptake of metallothionein by rat renal brush border membrane vesicles: inhibition by anti-megalin antibodies.

Rat renal cortical brush border membrane vesicles were isolated by magnesium precipitation techniques as described previously (32 Moestrup, 33 Hammond, 39 Batuman). The binding of metallothionein was investigated in the presence of 1000- to 2000-fold dilutions of

anti-cubilin or anti-megalin polyclonal antibodies that recognize the holoprotein (33 Hammond, 34 Sahali, 35 Sahali, 39 Batuman). Anti-NK1 peptide antibody was chosen as a control because it bound BBMV in the same titer as the antimegalin antisera. Binding of FluorX™ (Amersham) conjugated metallothionein was analyzed by flow cytometry using a FACStar Plus flow cytometer to collect data files of 2000 observations per sample. All antisera were used at 1:1000 dilutions, which represented peak binding on dilution curves.

Statistics.

Data are expressed as mean ± standard error of the mean throughout the manuscript. Statistical analysis was performed by analysis of variance and Bonferroni or Scheffe's post hoc comparison. Flow cytometry data were also analyzed by Kolmogorov-Smirnov summation statistics (47 Young).

RESULTS

.Molecular studies by surface plasmon resonance (SPR).

We studied cubilin and megalin separately by using SPR, immobilizing purified membrane-free samples of each receptor and studying its interaction with rabbit liver metallothionein class I (metallothionein-I). The dose dependent binding to megalin is shown in Figure 1a. The responses uniformly increased with dose over the concentration range 75-2400 ug/mL. The observed variations and noise are normal for the very low signal levels used to optimize a study of binding kinetics. Even at high metallothionein-I concentrations, >90% saturation was not achieved, and therefore some errors occurred in the fit. An approximate fit using the maximum (but non-equilibrium) responses obtained at each concentration yielded an

estimated dissociation constant of 9.8×10^{-5} M (Figure 1b). Repeated experiments consistently indicate the binding of approximately 0.7-0.9 moles of metallothionein-I per mole of megalin, consistent with 1 binding site. In contrast, no binding of metallothionein-I to cubilin was observed.

The binding shown in Figure 1 was specific for megalin and depended on metal ions but not on the metallothionein-I source. Omitting either Ca or Mg from the sample buffers abolished the binding; both appeared to be required. Samples of metallothionein-I from horse kidney and from rabbit liver provided nearly identical results.

The binding of metallothionein-I was not inhibited by several other ligands of megalin. Myeloma light chains (Klassen & Hammond, mss in preparation), for example, produced results that were simply additive, indicating non-competitive binding. Retinol-binding protein (48 Christensen) behaved similarly. Myoglobin bound to megalin, but was not competitive (Klassen, unpublished results). Commercial sources of β_2-microglobulin bound megalin erratically, making assessment of competitive binding with metallothionein-I impractical to assess with current protein properties and purities.

Interestingly, oligomerized metallothionein-I bound more effectively to megalin than did the monomer. Non-denaturing gel electrophoresis showed that over time, metallothionein-I forms trimers, tetramers, and even much larger oligomers. The binding of such molecules to megalin was significantly stronger. Owing to difficulties in purifying these oligomers, the actual binding constants for oligomers could not be determined with any precision. Qualitatively, compared to monomeric metallothionein-I, oligomeric metallothionein-I dissociated much more slowly and harsher conditions were required to dislodge it from immobilized megalin. Using the tetramer as a basis for calculations, one may estimate a 100-fold change in K_d (7×10^{-7} M).

Protein-receptor binding and antibody inhibition studies by flow cytometry.

The binding of fluorescent metallothionein-I to vesicles was readily detected (Figure 2). The addition of anti-megalin antibodies was able to displace nearly all bound metallothionein-I (>90%, $n = 5$, $p < 0.01$ by ANOVA and Scheffe, as well as on each individual run by Kolgomorov-Smirnov. Antibodies to cubilin or to the unrelated NK-1 receptor (a negative control) had little or no effect.

Cell culture studies by fluorescence microscopy.

Immortalized yolk sac cells from the Brown Norway rat (BN-16) cells were incubated with fluorescently labeled metallothionein-I at 37 °C. After 30 minutes, fluorescence microscopy revealed that much metallothionein-I could be found in the cells in a granular form, consistent with metallothionein-I uptake into endosomes. In order to follow cellular uptake more closely, we incubated BN-16 cells with labeled metallothionein-I at 4 °C and chased with unlabeled metallothionein-I for variable intervals. At 4 °C, metallothionein-I bound to the surface but did not enter the cells, while incubation at 37 °C afterward led to uptake. Colocalization with antibodies to the transferrin receptor, an early endosomal marker, indicated that metallothionein-I entered the early endosomes within 15 minutes but passed beyond them in less than 45 minutes.

Colocalization of metallothionein-I with both megalin and cubilin was demonstrated by using receptor antibodies in conjunction with a fluorescent secondary antibody. At 4 °C, megalin, cubilin, and metallothionein-I were co-localized on the surface, while after 15 min at 37

°C they had all migrated to the early endosomes (Figure 3, panels a-f). After 45 min little evidence for colocalization remained. No colocalization was observed with antibodies to giantin, an unrelated protein found in the Golgi apparatus and used as a negative control. While no detailed studies have yet been completed using confocal microscopy, early results with this higher resolution method confirmed that metallothionein-I and megalin or cubilin were co-localized. In panels g-i of Figure 3, one can see similar patterns of distribution for fluorescent metallothionein-I and antibody.

As a negative control, MDCK cells were examined for evidence of metallothionein-I uptake. These cells do not express cubilin or megalin, and in fact we found that they did not import metallothionein-I at all, demonstrating that ordinary membrane diffusion (of free dye or of conjugated metallothionein-I) cannot explain our results with BN-16 cells.

Inhibition of metallothionein-I uptake by small peptides derived from metallothionein-I.

We prepared a series of peptides spanning the sequence of rabbit liver metallothionein-I and used SPR to study their effect on the binding of metallothionein-I to megalin. Six 16-amino acid peptides, each overlapping its neighbors by 7 amino acids, were prepared as shown in Figure 4 (46 Kagi). Those peptides readily soluble in water were used for inhibition studies. The results of these initial qualitative studies are summarized in Table 1. Interestingly, peptides 3 and 4 bound quite tightly to megalin and also disrupted the binding of metallothionein-I. Although peptides 1, 2 and 6 contain cysteines they did not bind megalin, suggesting that the binding of peptides 3 and 4 is a specific interaction, rather than a nonspecific disulfide interaction between the peptides and megalin. Technical issues prevented direct confirmation; reduction with DTT denatured megalin and abolished the binding of all ligands. Because the

behavior of peptides 3 and 4 differed significantly from that of the other soluble peptides, we turned our attention to the overlap sequence these peptides have in common.

Table 1 Binding to megalin by peptides derived from metallothionein-I and interference with binding of the native protein.

Peptide #	Sequence	Binding to megalin	Competition with metallothionein-I for megalin binding
1	KMDPNCSCATGNSCTCA	No	No
2	GNSCTCASSCKCKECK	No	No
3	CKCKECKCTSCKKSCC	Yes	Yes
4	SCKKSCCSCCPAGCTK	Yes	Yes
5	CPAGCTKCAQGCICKG	Insoluble; no data	Insoluble; no data
6	CAQGCICKGASDKCSCCA	No	No

A peptide representing this overlap sequence, SCKKSCC, bound to megalin and also disrupted the binding of native metallothionein-I. The dose-dependent binding of this peptide is shown in Figure 5a. Its ability to affect the binding of metallothionein-I to megalin is apparent in Figure 5b, in which the binding of metallothionein-I decreased when coinjected with peptide. In contrast, peptides containing a lysine repeat but derived from unrelated ATPase or Apo A-I sequences had no apparent effect, producing instead responses that were essentially additive. The polybasic megalin ligand gentamicin bound megalin with affinity much lower than metallothionein-I, and showed no interference with metallothionein-I binding (Table 2).

Table 2 Binding to megalin by polybasic peptides and gentamicin, and interference with binding of native metallothionein-I.

Peptide or reagent	Sequence	Binding to megalin, compared with metallothionein-I	Effect on metallothionein-I binding to megalin
metallothionein sequence overlap of peptides 3 & 4[a]	SCKKSCC	Affinity ~ metallothionein	Competitive with metallothionein binding
v-ATPase β-subunit peptide with KK motif[b]	CLQKFEKKINQSPYEKR	Affinity << metallothionein	Additive to metallothionein binding
Apolipoprotein A1 peptide with KK motif[c]	ALEEYTKKLNTQ	Affinity << metallothionein	Additive to metallothionein binding
Gentamicin	-	Affinity <<< metallothionein	Additive to metallothionein binding

Peptide inhibition of metallothionein-I uptake.

All sequenced mammalian metallothionein-I proteins have 61 or 62 amino acids. The residues include 20 conserved cysteines and a high overall homology. The two forms of metallothionein-I used in these studies were derived from horse kidney and rabbit liver, which differ in fewer than 10 residues throughout their sequences. As such, similarities in their binding to megalin might be expected, but distinct differences would suggest a possible starting point in the design of peptide inhibitors. Metallothionein-I from horse kidney and rabbit liver, however, showed little difference in binding behavior by SPR.

We examined several ligands of megalin by using SPR in the hope that one of these might inhibit metallothionein binding, suggesting a shared binding site. Myoglobin, myeloma light chains and retinol-binding protein all produced additive results when co-injected with metallothionein-I, consistent with binding to different sites on megalin. Because megalin has

been established to have multiple binding sites (30 Saito), this result was not completely unexpected. Nevertheless, this result should be tested further. In one related study, reproducible demonstration of renal tubular uptake inhibition required the sensitivity of radiotracer methods in an intact cell system (44 Dreisbach). Such an approach will form a natural follow-up to the studies reported here, especially as we would like to confirm initial reports of the mutual inhibition of metallothionein-I and β_2-microglobulin in intact animals (20 Bernard, 21 Bernard).

Our initial dissection of the site in the metallothionein-I sequence critical for binding to megalin clearly implicates the hinge region. In lower species, metallothionein exists as two separate molecules, binding 3 and 4 heavy metal moieties. But in mammals and other higher organisms the two molecules have coalesced, joined by a hinge region centered on a highly charged lysine repeat. The hinge region sequence SCKKSCC is even more heavily conserved than the rest of the metallothionein-I sequence, being identical in virtually all known mammalian species, and all the various metallothionein isoforms in each species. This fact would explain our own observations that diverse metallothioneins bind megalin with the same kinetics, and may be important to ensure efficient re-uptake of diverse isoforms in the proximal tubule.

Studies using site directed mutagenesis have established the critical role of the conserved lysine repeat in the detoxification function of metallothionein in yeast (43 Cody, 44 Cody). Replacement of one or both lysines in the hinge or interdomain region is inconsequential to the structure and function of metallothionein unless both substituted residues are uncharged (44 Cody). However, our observations on other peptides and gentamicin suggest there are more structural requirements for a molecule to interfere with the megalin metallothionein interaction than simply charge. Other peptides containing a lysine repeat did not interfere, and the highly charged polybasic drug, gentamicin, which is a known ligand for megalin (29 Moestrup), also

did not interfere (Table 2). The failure to observe competition between metallothionein-I and other ligands may be an artifact of megalin isolation or ligand purity, or it may reflect the physiological condition. Recombinant production of metallothionein fragments dividing metallothionein at the lysine-lysine hinge yields intact α and β subunits (49 Atrian) These fragments still bind heavy metals (49 Atrian); if our prediction that they no longer bind megalin is borne out in future experiments, they may provide a reagent to deliver therapeutic heavy metals like gold or cisplatinum without renal uptake or toxicity.

In summary, this study provides three lines of evidence that megalin binds metallothionein, and that this is the predominant mechanism of uptake of metallothionein and its conjugated heavy metals in the kidney. The hinge region of metallothionein, based around the highly conserved lysine repeat, is the critical peptide sequence for the metallothionein–megalin-binding interaction. Metallothionein fragments or mutants that truncate or mutate the hinge region may prevent uptake of conjugated heavy metals and secondarily diminish or abolish heavy metal renal tubular damage.

Surface plasmon resonance analysis of binding of recombinant human MT subunits to megalin.

Iinitial attempts at recombinant production of metallothionein were characterized by very low yields or by a product consisting of several short cleavage fragments of the MT molecule. This problem can be solved making recombinant metallothionein subunits in *E. coli* using a GST fusion vector followed by thrombin cleavage to release the free metallothionein subunit, utilizing robust inert gas conditions at all feasible steps. The thrombin cleavage leaves three amino acids, specifically SCM derived from the C-terminus of the GST, on the N-terminus of the product. To understand our preliminary data we must understand that we postulate the critical binding site on

metallothionein to be the interdomain SCKKSCC region with SCK representing the C-terminus end of the β subunit, and KSCC the N-terminus start of the α subunit. The recombinant α subunit therefore has a conservative GST derived SCM substitution for SCK on its thrombin-cleaved N-terminus, leaving our postulated critical SCKKSCC sequence essentially intact.

Atomic absorption (ICP) analysis of the zinc content of the recombinant subunits proved them to be at the predicted heavy metal content to within the error of the methods (Table 3). The protein values represent the smallest scale production possible and up to 100 times this amount of protein can be produced in a single batch.

Table 3

Clone	Protein concentration/total yield by A_{280}	Zinc content as zinc/protein ratio by ICP	Predicted ideal zinc/protein ratio
MT-I full-length recombinant	$1.18*10^{-4}$ M 2.14 mg. total	6.73	7
MT-I α-subunit recombinant	$1.37*10^{-4}$ M 1.58 mg. total	3.93	4
MT-I β- subunit recombinant	$1.87*10^{-4}$ M 2.01 mg. total	3.01	3

SPR analysis of binding to megalin shows that, when corrected for the molecular weight of the protein fragments (n = 2 for each analysis, Zn and Cd results essentially identical), the recombinant full length clone bound 95% as well as the native protein, the α-subunit with intact conservatively substituted SCKKSCC region also bound around 94% as well as the native MT. However the β-subunit in which the SCKKSCC region is divided at the KK has binding reduced to 10% of the predicted value (Table 4).

Table 4

	% predicted binding by SPR
Native full length MT	100 (positive control)
MT-I full-length recombinant	95
MT-I α-subunit recombinant	94
MT-I β- subunit recombinant	10

We have found that oxidation and degradation of the product continues to be problematic despite vigorous use of degassing and performing procedures under inert gases. Hence, we propose to continue the recombinant approach with site directed mutagenesis which has worked well for us to date. We propose to produce α-subunits both in native sequence and the terminal highly charged K changed to the neutral amino acid Q. We cannot change a C without losing heavy metal binding[43,49] but may need to mutate the S in the SCKKSCC motif to further inhibit binding to megalin. We also propose to produce full length MT-I with the interdomain KK changed to QQ by site directed mutagenesis. This data proves that we have a recombinant metallothionein fragment which binds Cd and has a 90% reduction in megalin binding in hand, and we are aggressively exploring a systematic approach to even better reagents with less megalin binding.

In a further proof of principle experiment the recombinant mouse metallothionein fragments were used to compete with the uptake of fluorescently labeled metallothionein into BN cells (Figure 6), a rat immortalized placental epithelial cell line which expresses abundant cubilin and megalin. MT conjugated to the fluorophor fluorescein was incubated with BN cells for 2.5 hours, and the effects of a 25 fold excess of recombinant proteins examined. Following uptake in a 37°C, 5% CO2 incubator in 96-well plate wells, the cells were placed on ice, acid

washed to remove surface bound reagents, trypsinized off the plate surface and washed. Fluorescein-MT uptake was analyzed on a cell-by-cell basis by flow cytometry. The results shown in the Figure 6 show that compared to a non-fluorescent control (0 ± 0 arbitrary fluorescent units, n=6), the cell took up fluorescent-MT (125 ± 18, n=6, $p<0.01$). Both full length recombinant metallothionein (13 ± 1) and alpha subunit with SCMKSCC intact (5 ± 1) significantly inhibited uptake of fluorescent MT (n=6, $p<0.01$ ANOVA and Scheffe). The beta subunit recombinant in which SCKKSCC is disrupted had significantly less effect (64 ± 5, n=6, $p<0.05$). This provides another line of evidence for a roll of the intradomain SCKKSCC region in the megalin – metallothionein interaction and uptake of MT into megalin expressing cell lines.

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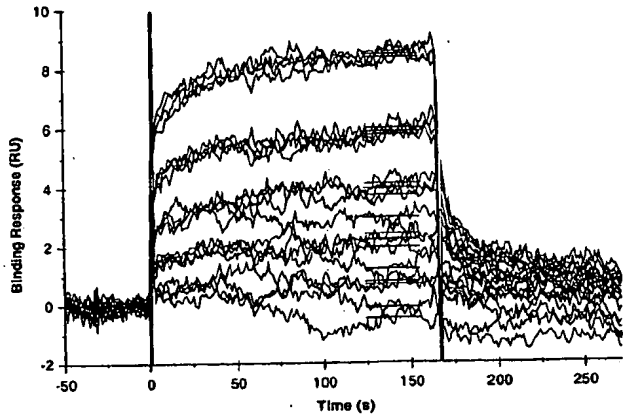
Therefore we CLAIM the following

1. Mutants of metallothionein that do not bind megalin.
2. Mutants of metallothionein that bind heavy metals but do not bind megalin.
3. Fragments of metallothionein that do not bind megalin.
4. Fragments of metallothionein that bind heavy metals but do not bind megalin.
5. A method of administering therapeutic metals that is comprised of, at least, the administration of fragments of metallothionein that do not bind megalin.
6. A method of administering therapeutic metals that is comprised of, at least, the administration of mutants of metallothionein that do not bind megalin.
7. A method of administering a therapeutic metal that is comprised of, at least, the administration of fragments of metallothionein that do not bind megalin.
8. A method of administering a therapeutic metal that is comprised of, at least, the administration of mutants of metallothionein that do not bind megalin.


Binding Response (RU)
Time (s)

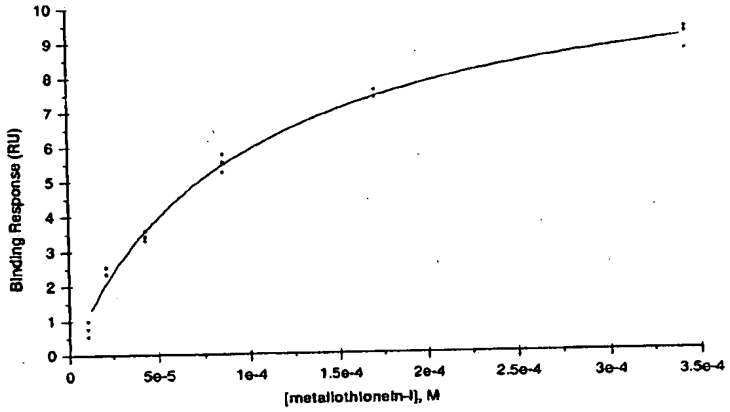

Binding Response (RU)
[metallothionein-I], M

Figure 1.

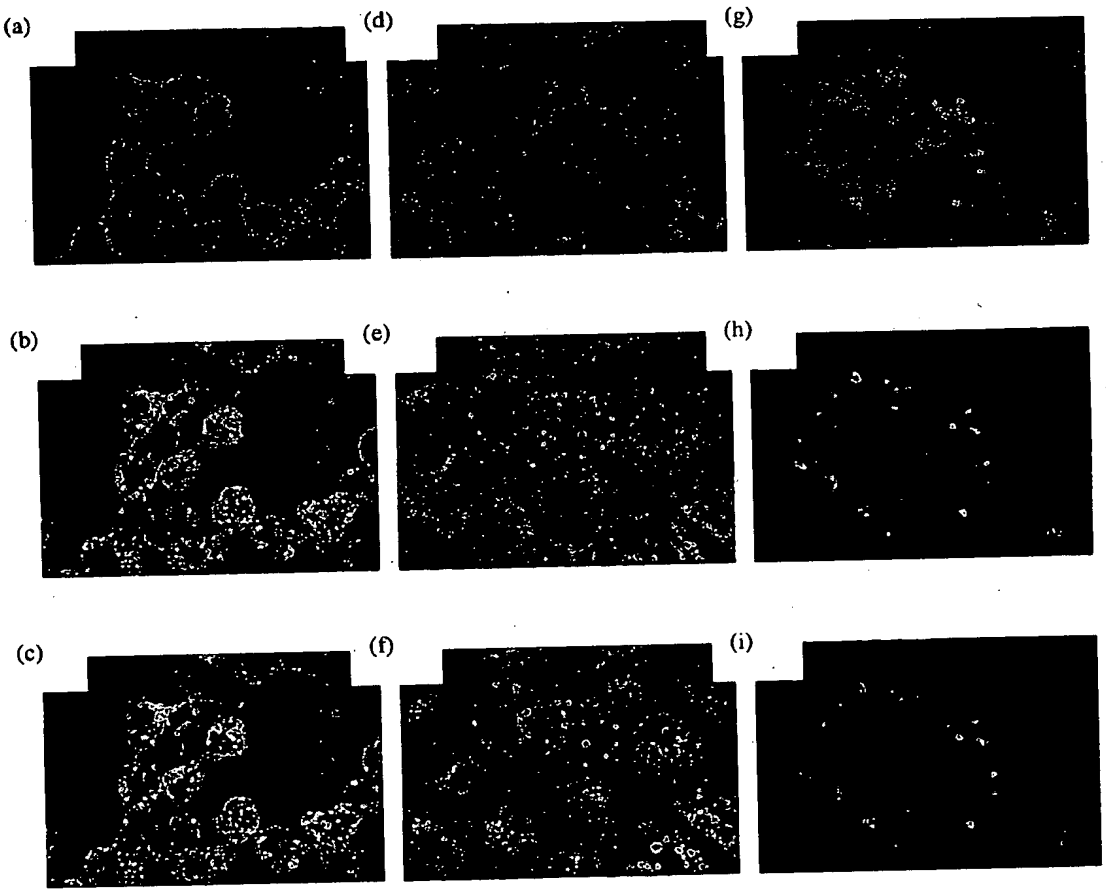


Figure 2.

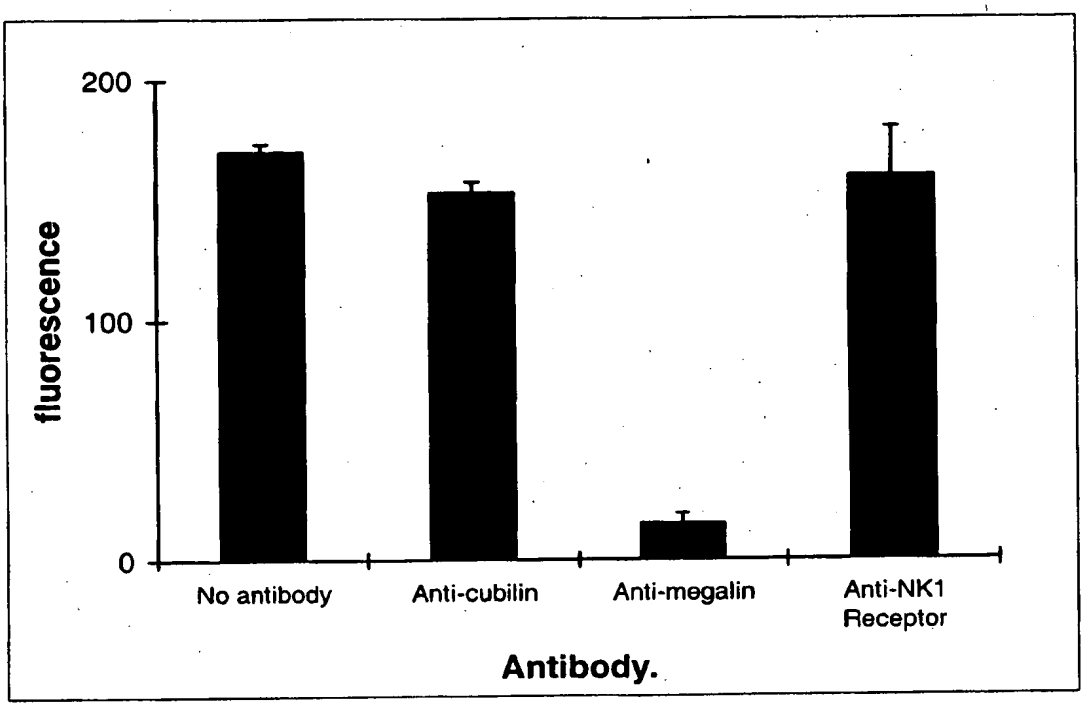
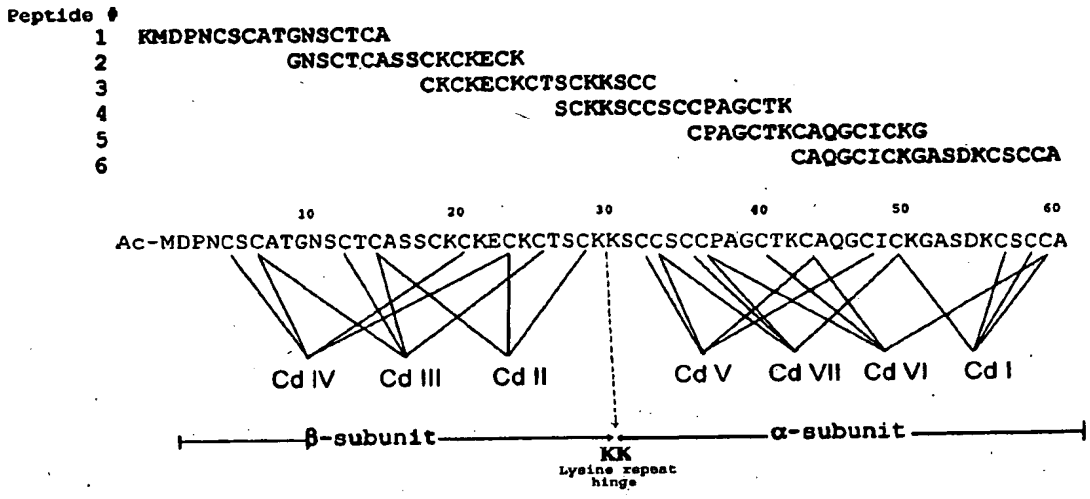

200
100
0
fluorescence
No antibody
Anti-cubilin
Anti-megalin
Anti-NK1 Receptor
Antibody.

Figure 3.



metallothionein-I sequence, binding sites, domains, and hinge

Figure 4.

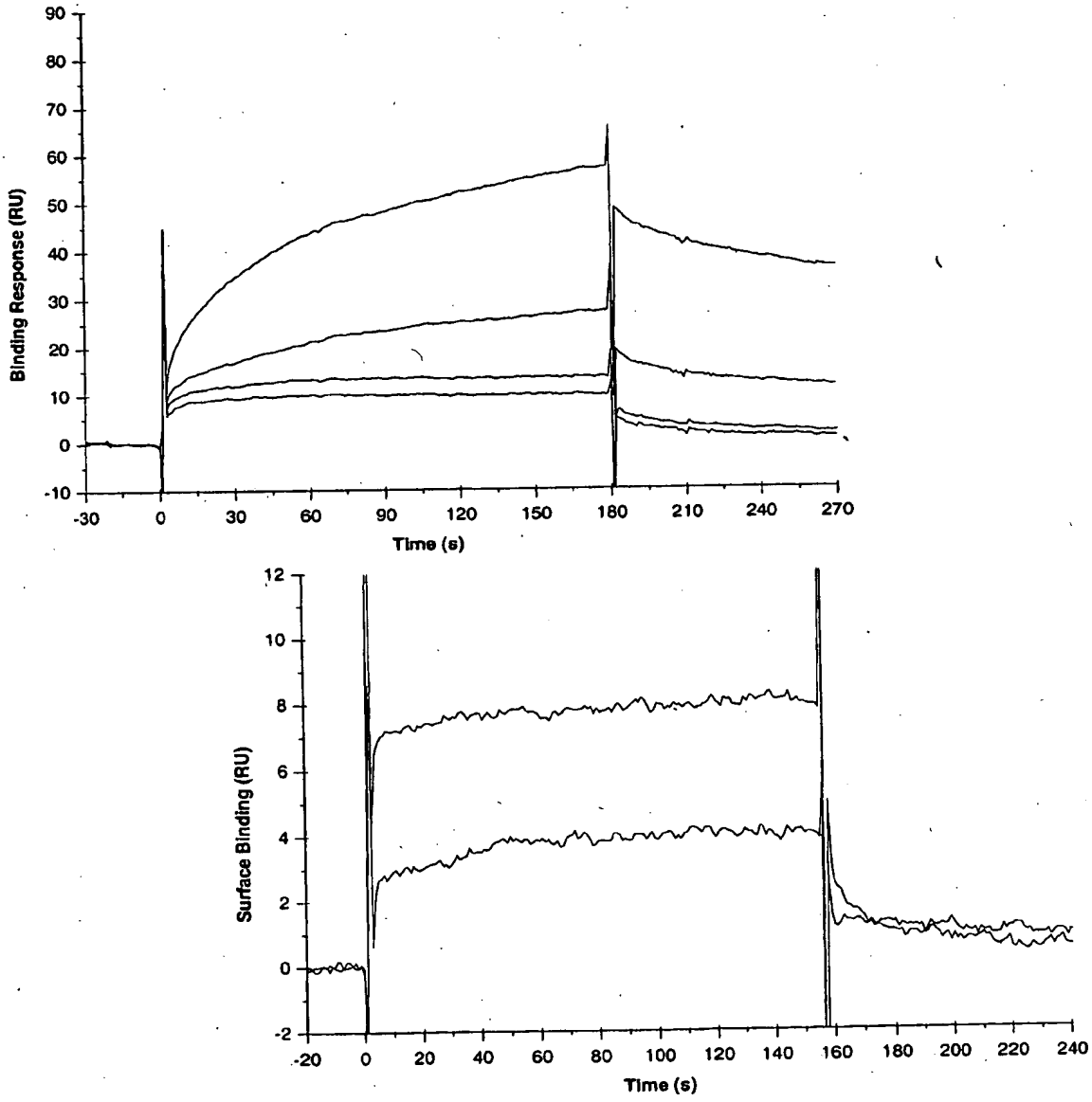

Binding Response (RU)
90
80
70
60
50
40
30
20
10
0
-10
-30
0
30
60
90
120
150
180
210
240
270
Time (s)
Surface Binding (RU)
12
10
8
6
4
2
0
-2
-20
0
20
40
60
80
100
120
140
160
180
200
220
240
Time (s)

Figure 5.

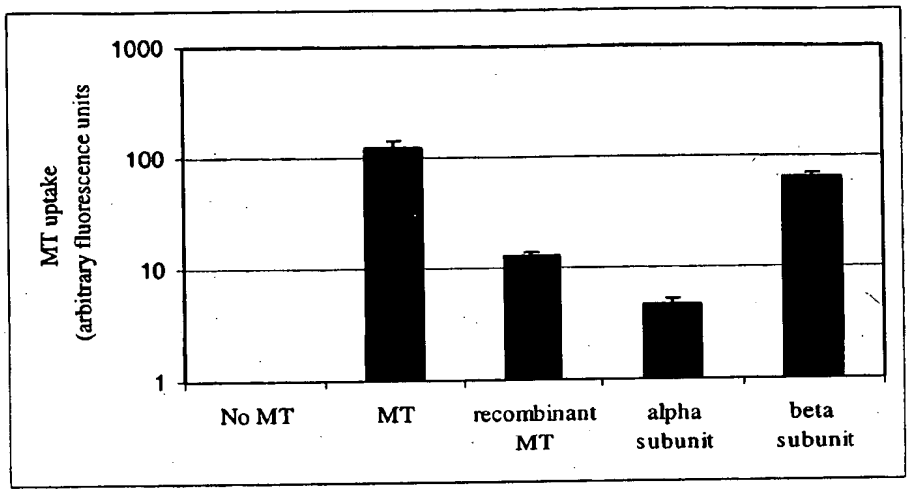

1000
100
10
1
MT uptake
(arbitrary fluorescence units
No MT
MT
recombinant MT
alpha subunit
beta subunit

Figure 6.